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Chronic Chagas disease: PCR-xenodiagnosis without previous microscopic observation is a useful tool to detect viable *Trypanosoma cruzi*

Miguel Saavedra[1], Inés Zulantay[1]*, Werner Apt[1], Gabriela Martínez[1], Antonio Rojas[1], Jorge Rodríguez[2]

[1] Laboratorio de Parasitología Básico-Clínico, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas.
[2] Escuela de Salud Pública. Facultad de Medicina, Universidad de Chile, Santiago, Chile. P.O. Box 427 - Santiago 3.

ABSTRACT

We evaluate the elimination of the microscopic stage of conventional xenodiagnosis (XD) to optimize the parasitological diagnosis of *Trypanosoma cruzi* in chronic Chagas disease. To this purpose we applied under informed consent two XD cages to 150 Chilean chronic chagasic patients. The fecal samples (FS) of the triatomines at 30, 60 and 90 days post feeding were divided into two parts: in one a microscopic search for mobile trypomastigote and/or epimastigote forms was performed. In the other part, DNA extraction-purification for PCR directed to the conserved region of kDNA minicircles of trypanosomes (PCR-XD), without previous microscopic observation was done. An XD was considered positive when at least one mobile *T. cruzi* parasite in any one of three periods of incubation was observed, whereas PCR-XD was considered positive when the 330 bp band specific for *T. cruzi* was detected. 25 of 26 cases with positive conventional XD were PCR-XD positive (concordance 96.2%), whereas 85 of 124 cases with negative conventional XD were positive by PCR-XD (68.5%). Human chromosome 12 detected by Real-time PCR used as exogenous internal control of PCR-XD reaction allowed to discounting of PCR inhibition and false negative in 40 cases with negative PCR-XD. Conclusion: PCR-XD performed without previous microscopic observation is a useful tool for detection of viable parasites with higher efficiency then conventional XD.

Key words: Chronic Chagas disease, PCR, *Triatoma infestans*, *Trypanosoma cruzi*, xenodiagnosis.

INTRODUCTION

Chronic Chagas disease is characterized by levels of circulating parasites far below the threshold of microscopic detection (Gomes et al., 2009). For this reason the conventional parasitological techniques with best results in this phase of infection are those which allow the amplification of *Trypanosoma cruzi*, such as xenodiagnosis (XD) or blood culture (Schenone, 1999; Castro et al., 2002). Molecular-based assays, in particular amplification by the polymerase chain reaction (PCR), is able to detect 2.5 parasite genome equivalents/mL of blood, showing greater sensitivity than XD and blood culture (Schijman et al., 2003; Britto, 2009). XD is a laborious procedure which uses the vector as a biological culture medium for the detection of *T. cruzi* infection in man and other mammals (Schenone, 1999). PCR in fecal samples (FS) of *Triatoma infestans* fed by conventional XD (PCR-XD) with microscopic investigation of *T. cruzi* has been applied in other studies (Zulantay et al., 2011). We decided to evaluate whether it would be possible to eliminate the microscopic stage of XD, in order to optimize the technique and minimize the risk of accidental infection when handling FS of possibly infected triatomines.

MATERIALS AND METHODS

Population study

150 persons were selected with chronic Chagas disease confirmed serologically by ELISA (Chagatek ELISA, Biomérieux, France) and IFI-IgG (in-house), with ages between 18 and 61 years, average 50 years. All of them are members of rural and urban communities from the provinces of Choapa and Limarí, IV Region of Coquimbo, Chile and accepted participation in this study under informed oral and written consent approved by the Ethics Committee of the Faculty of Medicine of the University of Chile (Resolution 046/August 2009). All patients are immunocompetent and were studied between 2009 and 2011. At the moment of this study all had the condition of cases not treated with anti-trypanosome pharmacological therapy.

Conventional xenodiagnosis and PCR applied to fecal samples of Triatoma infestans

The colony of *T. infestans* used in XD has been maintained in our laboratory for 50 years, fed on chickens, which are normally refractory to *T. cruzi* (Schenone et al., 1974). XD was applied using two cylindrical wooden boxes each containing seven uninfected third or fourth instar nymphs of *T. infestans* starved for a period of 3-4 weeks. The insects were fed for 20-30 min on the arm of each of the patients. The cages of XD were maintained at 27 ºC and 70% relative humidity without further feeding. After 30, 60 and 90 days of incubation, the FS of triatomines fed on each patient were obtained by abdominal compression in a biologically secure hood and divided into two parts: one of them was placed directly on slides with 100 µL of PBS pH 7.2 for examination under an optical microscope at 40x to detect mobile trypomastigotes or epimastigotes of *T. cruzi* (conventional XD). At the end of the microscopic observation this biological

* Corresponding author: Dra. Inés Zulantay, Laboratorio de Parasitología Básico-Clínico, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas Facultad de Medicina, Universidad de Chile, P.O. Box 427 - Santiago 3. Santiago, Chile. E-mail: izulanta@med.uchile.cl, Telephone: +56 2 9786753, Fax: 56 2 9786122.

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material was eliminated under bio-safety conditions. The criteria to determine a negative XD was the absence of mobile forms of *T. cruzi* in the three periods of microscopic observation (30, 60 and 90 days). The second part of fresh FS was deposited directly (without performing microscopic observation) in an Eppendorf tube containing 500 μL of PBS pH 7.2. This sample was immediately incubated for 15 min at 98 ºC to cleave the kDNA network of *T. cruzi* and then centrifuged for 3 min at 1200 g (Microspin 12S Centrifuge, DuPont Co., USA) to obtain a supernatant free of fecal material, which was transferred to a new Eppendorf tube and maintained at -20 ºC until use. This process was repeated in all the cases in study for each period of incubation, independently of XD results.

Finally, a pool of supernatants of 30, 60 and 90 days was prepared and stored at -20 ºC until extraction and purification of DNA for PCR (Zulantay et al., 2011). In total, 900 microscopic observations were performed, two for patient in each period of study. However, the XD was considered positive when mobile forms of *T. cruzi* were observed, at least in one of three period of study. Finally, 150 pools of FS for PCR-XD reactions were prepared, one for each patient in study (Figure 1).

Exogenous internal control for negative PCR-XD reactions

We used human blood DNA as an exogenous internal control (EIC) with chromosome 12 (X12) detected by Real-time PCR as described Bravo et al., (2012). X12 primers were designed for application of AmplifX v.1.5.4 (N.Jullien) software, and then compared with Nucleotide BLAST (National Library of Medicine) to discount any other unspecific amplification. A 50 ng equivalent of human blood DNA negative for *T. cruzi* was added to each pool of supernatants of all samples in study. Real-time PCR was performed only in cases of negative PCR-XD to discard inhibition and false negative reactions (n=40).

DNA purification of fecal samples

DNA purification was performed using an initial volume of 100 μL of the FS supernatant pool of each patient with the commercial kit FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Australia), modified by omitting the cell lysis step and washing the column twice with 50 μL of elution buffer previously incubated for 10 min at 56 ºC, obtaining a final volume of 100 μL of purified DNA. Samples were maintained at -20 ºC until amplification.

kDNA PCR assay

The PCR-XD was performed in triplicate using oligonucleotides 121 and 122, which anneal to the four conserved regions present in *T. cruzi* minicircles (Degrave et al., 1988), to obtain 330 bp amplicons. Each sample was tested in a final volume of 20 μL including 5 μL of extracted DNA. The final concentrations of the reagents were as follows: 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer and 1 unit of GoTaq DNA polymerase (Promega Corp.,USA). The amplification program was performed in a TC 412 thermal cycler (Techne, UK), and included initial denaturation at 98 ºC for 1 min and 64 ºC for 2 min; 33 cycles of 94 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min, and a final incubation at 72 ºC for 10 min. Each experiment included two negative PCR controls: water instead of DNA and DNA of non-chagasic patients. As positive control purified DNA of *T. cruzi* Tulahuén strain was used. Amplification products were analyzed by electrophoresis in a 2% agarose gel and visualized after staining with GelRed™ (Biotium Inc., USA). 5 μL Bench Top 100 bp DNA ladder (Promega Corp.,USA) was incorporated. A positive result for PCR-XD was the presence of a 330 bp band specific for trypanosome minicircles.

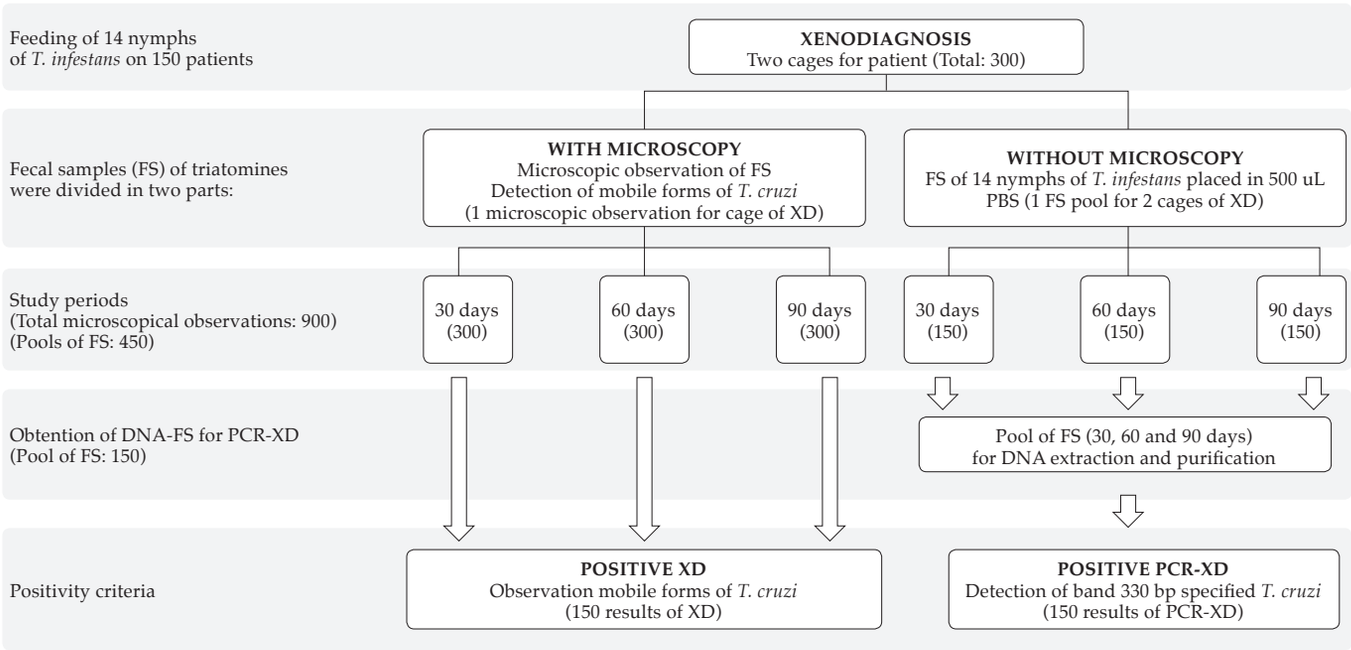

Figure 1. Algorithm of detection of *Trypanosoma cruzi* in 150 patients with chronic non treated Chagas disease by XD (with microscopy) and PCR-XD (without microscopy) in fecal samples (FS) of *Triatoma infestans.*

Real-time PCR for chromosome 12 (X12)

The N1X12 forward (5′-AGCTGGCTAGACTGTCAT-3′) and N2X12 reverse (5′-CTTTGCCGTTGAAGCTTG-3′) primers were used at a concentration of 1 μM (Integrated DNA Technologies) and the Real-time PCR reaction was carried out using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies), Molecular Biology Grade water (Mo Bio Laboratories, Inc.), and 2 μL of DNA isolated from FS with or without EIC, in a final volume of 20 μL. The thermal profile included 3 min of pre-incubation at 95 °C and 40 amplification cycles (95 °C for 5 sec, 65 °C for 15 sec and 72 °C for 10 sec). The measurement of emitted fluorescence was performed at 72 °C at the end of each cycle. After all the amplification cycles a melting curve was run. The results were analyzed with the MxPro v4.1 (Agilent Technologies) software.

Statistical analysis

To determine the confidence intervals a Normal curve with 95% confidence was used. The McNemar test was applied to compare the proportions of positive and negative samples determined by conventional XD and PCR-XD.

RESULTS

25/26 cases (96.2%) were positive by conventional XD with microscopy and PCR-XD without microscopy, while 85/124 cases (68.5%) were negative by conventional XD with microscopy and positive by PCR-XD without microscopy. The percentage of positive PCR-XD cases without microscopy was significantly greater than the percentage of positive conventional XD case with microscopy (McNemar Chi^2=82.05; p<0.0001). The 95% confidence intervals of PCR-XD and XD positive were 65.9% and 80.1%, and 11.2% and 23.4%, respectively. Of the 150 cases evaluated by PCR-XD without microscopy, 40 were negative (27%), one had positive XD and the others had negative conventional XD (Table 1). All the negative cases by conventional XD and PCR-XD at 30, 60 and 90 days (n=39) were not submitted to new studies because it has been demonstrated that the mortality of the insects increases drastically after 90 days (87%) (Schenone et al., 2000). In the 40 cases with negative PCR-XD, EIC showed amplification of X12 detected by Real-time PCR. This result allowed us to discard PCR-XD inhibition and false negatives in these samples.

DISCUSSION

The PCR technique has motivated enthusiasm and influenced researchers to consider not using the traditional parasitological procedures commonly employed in the diagnosis of *T. cruzi*, among them XD and blood culture for detecting *T. cruzi* (Amato Neto, 2012). Certainly, PCR has been widely applied for the detection of *T. cruzi* DNA during the 20 last years in different types of biological samples (Britto et al., 1995; Botto-Mahan et al., 2005; Virreira et al., 2006, Burgos et al., 2008) and in follow-up studies of treated acute and chronic Chagas disease cases (Britto et al., 2009; Apt, 2010; Aguiar et al., 2012; Noya et al., 2012). The major advantages of conventional XD and blood culture are their potential to confirm the presence of viable *T. cruzi* and to isolate the parasite. This information is especially important to evaluate chemotherapeutic efficacy in treated cases of Chagas disease to establish whether the infection has healed or not (Amato Neto, 2012). In the present study we used the capacity of the biological vector to enable *T. cruzi* multiplication and differentiation in the tract of triatomine insects (Kollien and Schaub 2000) and we evaluated if it is possible to eliminate the microscopic phase of conventional XD and apply directly PCR in FS of *T. infestans* fed on chronic chagasic patients. PCR-XD was concordant in 25 out of 26 cases with positive XD and was more sensitive than conventional XD since 68.5% of PCR-XD positive cases were obtained of XD negative cases. The 39 cases with negative XD and PCR-XD probably correspond to patients with very low parasitemia levels (31.5% of the cases), under the limit of detection of PCR and XD methods (2.5 parasite genome equivalents/mL of blood for PCR and theoretically, 1 viable parasite in the blood of mammalian hosts ingested for the triatomines used in the XD). In these negative cases, was discarded the PCR inhibition and false negatives by Real-time PCR X12 positivity. A case with positive conventional XD and negative PCR-XD was probably due to the loss of parasite DNA of the FS during the extraction process, which could be corrected if in the future all the FS is used directly only for PCR-XD. Thus PCR-XD without previous microscopy could be used especially to evaluate persistence or clearance of treated chronic Chagas disease in patients follow-up. The higher

TABLE 1

Results of conventional XD (with microscopic observation) and PCR-XD (without microscopic observation) of the same samples of *Triatoma infestans* (Total samples: 300) 30, 60 and 90 days of incubation after feeding on 150 patients with chronic Chagas disease

		Without microscopy		
		PCR-XD positive* (110) 73%	**PCR-XD negative (40) 27%**	**Nº samples 150**
With microscopy	**XD positive* (26) 17.3%**	25 (96.2%)	1 (3.8%)	-
	XD negative (124) 82.7%	85 (68.5%)	39 (31.5%)	-
	Nº samples 150	-	-	Total samples 300

* Mc Nemar Chi2=82.05; p=0.0001

sensitivity and lower risk of accidental transmission of Chagas disease by contact of skin and/or mucosa with FS which may contain infective metacyclic trypomastigotes of *T. cruzi* (Schaub, 1989), constituted its advantages.

It is important to say that though XD is a valuable tool for the detection of viable *T. cruzi*, it is considered archaic (Tarleton and Curran, 2012) and its application in the parasitological diagnosis of human Chagas disease needs approval of ethical committees. It may provoke allergic reactions in some patients, requires having colonies of triatomines available and trained personnel. Additionally, studies of the evaluation of efficacy of drugs in the treatment of chronic Chagas disease with conventional negative XD does not necessarily indicate absence of the parasite, since studies of XD of chagasic patients or experimentally infected triatomines show different rates of infection, including insect vectors with zero parasites (Castro et al., 1983; García et al., 2010). Finally, not all parasites survive in the inhospitable gut environment of the insect vector (García et al., 2010). ¿Will it be these factors and information which leads to the disappearance of the application of conventional XD in the study of Chagas disease? It is possible that in the near future, XD may be replaced by other techniques with greater sensitivity, specificity and consensus of the scientific community.

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